Pharmacokinetics and Treatment Efficacy of Camptothecin Nanocrystals on Lung Metastasis

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ABSTRACT: Cancer metastasis is difficult to treat, and its outcome becomes dreadful for a patient. Lung is a major metastatic site for many types of cancers, and the need for finding effective treatment for lung metastasis cannot be overemphasized. In a previous study, we showed that camptothecin nanocrystals demonstrated greater anticancer efficacy and achieved significantly higher concentration in lungs than a conventional, solution-based formulation. In this study, we further determined the pharmacokinetics of camptothecin nanocrystals in rats and investigated treatment efficacy in mice against metastatic lung tumors. The results show that camptothecin nanocrystals were capable of eliciting greater antimetastatic efficacy and achieve a longer survival time in the murine model compared with camptothecin salt solution. The study suggests that using engineered, solid nanoparticles may be a feasible approach in the treatment of lung cancer and lung metastatic cancer.

KEYWORDS: camptothecin, pharmacokinetics, metastasis, nanocrystals, drug delivery

INTRODUCTION

Cancer presents a great challenge to human health. In both developed and developing countries, cancer has become one of the leading causes of death. Cancer-related death generally results from eventual tumor-cell invasion into major organs and tissues. Survival at the metastatic stage is grim, and five-year survival rates of various cancers have not been improved much over last 20 years.1,2 Chemotherapy is mostly used for treatment, but the efficacy with existing chemotherapeutic regimens appears to be stagnant.3 Systemic, adverse effects are commonly associated with current chemotherapeutic systems, considerably limiting the treatment efficacy.

It is generally believed that tumor cells travel from the primary site via the circulation, attach to a suitable microenvironment, and thrive.4 As such, lung, liver, and bone are typical secondary sites for metastasis.4,5 Metastatic lung cancer is particularly difficult to treat.6 Prognosis remains dismal with the five-year survival rate less than 5% and a median overall survival of about one year.7 Developing new molecules and strategies to target cancerous sites are desired and have been very actively pursued.4,8 Passive targeting strategies that aim to take advantage of the enhanced permeation and retention (EPR) effect9 are ineffective when treating small and minimally vascularized metastases.4 Interestingly, it has been shown that particles larger than 300 nm, when given intravenously, can be more effective in targeting the lungs.10,11 This suggests a potentially effective way to target lung cancer including metastases by developing and utilizing nanoparticles in the range of a few hundreds of nanometers.

Our previous study tested the antitumor efficacy of camptothecin (CPT) nanocrystals in a xenograft murine model.12 Originally isolated from the wood and bark of Camptotheca acuminata in the 1970s, CPT is a topoisomerase I inhibitor.13 It has demonstrated to be effective against many types of tumors, but its poor water solubility and chemical instability limit its clinical application. At physiological pH or above, the lactone ring of CPT, necessary for its anticancer activity, is inclined to open up (Figure 1), rendering the drug much less useful but highly toxic and presenting a major hurdle to treat cancer patients.14 In our study, needle-shaped CPT nanocrystals were produced by solution crystallization.12 When given intravenously via tail-vein injection, the nanocrystal formulation achieved a much higher concentration in the lung than the drug salt solution (e.g., 1436.7 vs 88.9 ng per gram of
lung tissue 30 min after injection). On the other hand, our studies of paclitaxel nanoparticles, which were much smaller rod-shaped crystals, showed significantly less accumulation in the lungs after being injected intravenously to mice. It was thus suspected that, because of the relatively larger particle size—compared with the typical size range thought to be effective to exploit the EPR effect—and the long needle shape, the CPT nanocrystals were significantly entrapped in lung capillaries. This prompted the current study, reported here, to investigate the potential of using the CPT nanocrystals for treating lung metastases, including pharmacokinetics in rodents and treatment efficacy in a lung metastasis murine model of human breast cancer.

### MATERIALS AND METHODS

#### Materials.
Camptothecin was purchased from Chengdu Yuancheng Biotech Co. (Chengdu, China). D-Luciferin potassium was a product from Shanghai Sciencelight Biology Science & Technology Co. (Shanghai, China). Hoechst 33258 was obtained from Molecular Probes Inc. (Eugene, OR, USA). In situ cell death detection kit and TMR (tetramethylrhodamine) red were purchased from Roche Diagnostic GmbH (Mannheim, Germany). Cell culture media Roswell Park Memorial Institute medium (RPMI-1640), penicillin, streptomycin, and trypsin were obtained from M&C Gene Technology (Beijing, China). Fetal bovine serum was purchased from GIBCO, Invitrogen Corp. (Carlsbad, CA, USA). All other solvents and reagents were of analytical grade and used as received.

#### Preparation of CPT Salt Solution.
A sample of 150 mg of CPT was dissolved in a mixture of 15 mL of 1 M NaOH and 6 mL of propylene glycol at 60 °C. Once CPT was dissolved, 25 mL of water was added to the mixture, and pH was adjusted to 7 with 3 M HCl. The solution was sterilized by filtration. The final concentration after filtration was about 3 mg/mL. It was further diluted with 0.9% saline to 1.5 mg/mL prior to the animal test (to achieve an equivalent dose of 7.5 mg/kg).

#### Preparation and Characterization of CPT Nanocrystals.
CPT nanocrystals were produced by antisolvent precipitation augmented by probe sonication. Briefly, 0.5 mL of dimethyl sulfoxide solution of 2 mg/mL CPT was injected into 10 mL of water at a pH of 4 at room temperature. The solution was subjected to sonication (JY92-2D, Xinzhi Biotechnology Co., Ningbo, China) with power and duration set at 400 W and 3 min, respectively. The final product was filtered with a 50 nm polycarbonate membrane filter paper, and the filtrate was rinsed with water several times before it was eventually resuspended in water at a pH of 4.

The particle size and surface charge of the CPT nanocrystals were measured by dynamic light scattering (DLS) with a Malvern Zetasizer Nano ZS (Malvern Co., UK) in water at a pH of 4 at 25 °C. Particle morphology was investigated by a scanning electron microscope (SEM; JSM-5600LV, JEOL Ltd., Japan). Samples were prepared by filtering and collecting CPT nanocrystals on a 50 nm polycarbonate membrane paper which were then air-dried and sputter-coated with a conductive layer of gold palladium (Au/Pd) for 1 min.

#### Pharmacokinetics of CPT Nanocrystals.
Male Sprague–Dawley rats were obtained from Peking University Health Science Center with a body weight of 200 ± 20 g. Animals were housed at 25 °C and 55% of humidity under natural light with free access to food and water. The animals were randomly divided into two groups (n = 6) and fasted for 10 h but allowed water ad libitum prior to particle injection. CPT salt solution or nanocrystal suspension (0.1 mL) was injected intravenously to each animal via the tail vein at a single dose of 1.5 mg/kg. Subsequently, blood samples (0.5 mL) were collected into heparin-containing tubes at 0, 0.0833, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h, respectively, from each animal. Plasma was extracted by centrifugation at 10 000 rpm for 10 min and stored at −20 °C for further processing. Handling and surgical operations of the animals were approved by the Institutional Animal Care and Use Committee (IACUC) at Peking University.
To analyze the drug concentration in rat plasma samples, 200 μL was removed from a thawed sample and placed in a 1.5 mL plastic tube. Then, 20 μL of 3 M hydrochloric acid was added, and the solution was vortexed for 2 min. After allowing to settle for 2 h, the solution was mixed with cold acetonitrile (200 μL) and vortexed for 5 min followed by centrifugation at 10 000 rpm for 10 min. Supernatant (20 μL) was collected and used for chromatographic analysis.

CPT concentrations in the rat plasma were quantified by a Shimadzu high-performance liquid chromatography (HPLC) system equipped with a fluorescence detector (λ<sub>ex</sub> = 375 nm and λ<sub>em</sub> = 435 nm). A reverse-phase C-18 column (5 μm, 150 mm × 4.6 mm, Agilent Co.) was used and eluted at a flow rate of 1.0 mL/min at 35 °C by the isocratic mobile phase, which was made of 35% acetonitrile and 65% acetic acid aqueous solution (0.1%).

**Metastasis Inhibition.** A metastasis animal model was established in female BALB/c nude mice. Mice were obtained from Peking University Health Science Center with an average body weight of 20 ± 2 g and kept at 25 °C and 55% of humidity under natural light with free access to food and water. Highly metastatic human breast cancer cells that expressed detectable luciferase, MDA-MB-231/Luc, were obtained from the Peking University Medical and Health Analysis Center (Beijing, China). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL

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**Figure 3.** Chromatograms of blank rat plasma (A), blank rat plasma spiked with camptothecin at a concentration of 20 ng/mL (B), and plasma sample obtained 2 h after intravenous administration of camptothecin nanocrystals (C).
streptomycin at 37 °C in 5% CO₂ atmosphere. To induce metastasis in the lung, 2 × 10⁶ MDA-MB-231/Luc cells were inoculated via the tail vein of the nude mice. All animal experimental procedures were approved by the IACUC at Peking University.

To determine the metastasis inhibition, mice were randomly divided into three groups (12 animals in each group) 14 days after the injection of MDA-MB-231/Luc cells. The groups received one of three respective treatments: Group 1 received saline, Group 2 received CPT salt solution (CPT-Na), and Group 3 received CPT nanocrystals (CPT-NCs). Each animal received one injection, at a dose of 7.5 mg/kg spaced three days apart for both CPT treatments, which was half of the maximum tolerated dose of CPT.16 Approximately 4 h after the final injection, animals were administered intraperitoneal d-luciferin (150 mg/kg in phosphate-buffered saline, PBS) and observed 10 min later by bioluminescence imaging (IVIS Spectrum 200, Perkin-Elmer Co., MA, USA). The bioimaging exposure was 5 min at constant binning. Lung metastasis was then characterized by the signal intensity, which was measured as the sum of all detected photon counts less the background luminescence in the same ROI (region of interest) from a mouse in the control group, as in the unit of photons/second-cm²-steradian (p/s·cm²·sr).

In addition, two mice from each of the treatment group were sacrificed and the tumor samples were used for terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) analysis.17 Specifically, tissue samples of MDA-MB-231/Luc tumors were frozen in OCT embedding medium, cut into 8-μm-thick sections, and fixed in 4% (v/v) paraformaldehyde for 10 min at room temperature. The sectioned samples were washed with PBS three times and incubated with 0.1% (v/v) Triton X-100 (Sigma Co.) for 6 min on ice. TUNEL reaction mixture was then added to the tissue sections, which then incubated in the dark and under humidified atmosphere at 37 °C for 1 h. The samples were washed three times with PBS and treated with Hoechst 33258 (Molecular Probes Inc., OR, USA) for 30 min prior to microscopic analysis by a laser scanning confocal microscope (LSCM, Leica, TCS SP5, Germany).

**Tumor Survival.** The same MDA-MB-231/Luc tumor-bearing mouse model, discussed above, was used. Mice were randomly divided into three groups and received the same respective treatments of saline, CPT salt solution, and CPT nanocrystals. Mortality and morbidity, as an end point, was assessed every day for 46 days at which point all animals had succumbed to the cancer.

**Statistics Analysis.** All quantitative measurements were repeated at least three times and data were reported as mean plus or minus (±) the standard deviation (SD). One-way analysis of variance (ANOVA) was performed to determine the significance among treatment groups followed by Bonferroni’s post hoc test. Survival was assessed by Kaplan–Meier method and log-rank test. A p-value smaller than 0.05 was considered to be significant, and a p-value smaller than 0.01 was considered as highly significant.

# RESULTS AND DISCUSSION

CPT nanocrystals that were produced had good stability. They were stable over 6 months at 4 °C, and the crystal morphology did not change significantly.12 The crystals had an average particle size, measured by DLS, of 250 ± 16 nm with a polydispersity index (PDI) of 0.214 ± 0.004 (Figure 2A). SEM micrographs confirm that the nanocrystals have a rod or needle shape with the particle size—the longest dimension—ranging from 200 to 700 nm (Figure 2B) and an aspect ratio of approximately 10:1. TEM confirmed the size and shape of the nanocrystals (data not shown). The underestimated particle size determined by DLS was likely due to the irregular shape of the nanocrystals.

CPT concentrations were measured in blank rat plasma, blank rat plasma spiked with camptothecin at the concentration of 200 ng/mL, and rat plasma sample taken at 2 h after intravenous administration of camptothecin at the dose of 1.5 mg/kg (Figure 3). It is apparent that no interference from endogenous substances in the plasma was observed with the peak of the drug, validating the HPLC method where the limit of quantification (LOQ) and limit of detection (LOD) were 1.0 and 0.25 ng/mL, respectively, and R² of the linear regression between 1.0 and 500.0 ng/mL was 0.9988. The CPT peak was sharp and symmetrical with a good baseline resolution and minimal tailing. The retention time of CPT was 8.4 min.

Based on the HPLC analytical method, plasma concentrations of CPT in rats at different time points were analyzed after the animals received CPT salt solution or nanocrystals. The kinetic profiles (Figure 4) could not extend beyond 4 h due to drug concentrations too low to be detected by the analytical method. The plasma CPT level after administration of CPT salt solution was much higher at beginning when compared with that of the CPT nanocrystals, but it decreased very rapidly with time losing 97% from the blood after 30 min. While the plasma level of CPT nanocrystals was much lower during first 5 min, it slowly decreased within 30 min.

Pharmacokinetic characteristics of the two treatments were calculated (Table 1). It is shown that, compared with CPT salt solution, the area under the curve (AUC) of CPT nanocrystals was lower, but the distribution half-life (t½α) was higher, suggesting prolonged circulation by the nanocrystals. There was a trend toward higher elimination half-life (t½β) and MRT, but these were not statistically different between the CPT-NCs and CPT-Na. It seems to be plausible that because of the poor solubility, CPT nanocrystals slowly dissolved and released free drug molecules. The slow dissolution of the nanocrystals may in fact help delay the ring-opening reaction of the drug as well as potentially reduce the drug binding to with human serum albumin (HSA), which is also known to promote the lactone hydrolysis.13 Previously, we have observed the accumulation of similar nanocrystals in the lungs of animals with an ectopic breast tumor.12 If particles are accumulating in the lung, the clearance

![Figure 4](https://dx.doi.org/10.1021/mp4004018/mol-2014-126-233)
from blood in the first hours would not distinguish between systemic clearance and lung accumulation. However, it would be expected that, if nanocrystals accumulate in the lung, the blood level of CPT would either remain at a low level or recover to a measurable level. We did not observe a measurable concentration of CPT in the blood for the 24 h of the experiment. It is possible that we missed a later increase in blood levels that would suggest a depot is present. Thus, with respect to extending blood circulation, the CPT nanocrystals could offer significant advantages.

The antimetastasis efficacy of CPT nanocrystals was evaluated in mice by monitoring the tumor metastasis burden and survival rate. Bioluminescence imaging (BLI) was used to assess the therapeutic efficacy in the real-time and noninvasively. Because MDA-MB-231 cells express luciferases, the bioluminescent intensity of the cells can be used to quantify the survival of the cancer cells. The method to use BLI and luciferase-expressing cancer cell lines has been actively employed to study cancer treatment including metastasis of lymph nodes, lungs, bone, and soft tissues. Nude mice bearing lung metastasis tumor of breast cancer that were administered saline vehicle, CPT salt solution, and CPT nanocrystals, respectively. The bioluminescent intensities from the animals were quantified and summarized (Figure 5B). Among the treatment groups, the CPT nanocrystals achieved a better control of tumor metastasis, as evidenced by the lowest luciferase activities observed at the end of treatment (day 6). Compared with the control group, the CPT salt solution achieved a reduction in the intensity of 55% and the nanocrystals by 90%.

Tumor tissues from the animals at the end of treatment were further analyzed, and the extent to which tumor cells underwent apoptosis was assessed by TUNEL staining (Figure 6).

Table 1. Pharmacokinetic Metrics of Camptothecin in Rats after Intravenous Administration of Camptothecin Nanocrystals (CPT-NCs) and Camptothecin Salt Solution (CPT-Na) at the Dosage of 1.5 mg/kg (Mean ± SD, n = 6)\textsuperscript{a}

<table>
<thead>
<tr>
<th>pharmacokinetic metrics</th>
<th>CPT-Na</th>
<th>CPT-NCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2\alpha}$ (h)</td>
<td>0.11 ± 0.02</td>
<td>0.23 ± 0.03\textsuperscript{a}</td>
</tr>
<tr>
<td>$t_{1/2\beta}$ (h)</td>
<td>2.86 ± 5.71</td>
<td>12.76 ± 15.86</td>
</tr>
<tr>
<td>Cl (mL·h$^{-1}$)</td>
<td>755.64 ± 196.25</td>
<td>1896.76 ± 413.65\textsuperscript{b}</td>
</tr>
<tr>
<td>AUC (h·ng·mL$^{-1}$)</td>
<td>2104.07 ± 573.69</td>
<td>829.79 ± 217.87\textsuperscript{b}</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>1.39 ± 2.68</td>
<td>2.83 ± 3.48</td>
</tr>
</tbody>
</table>

\textsuperscript{a}These results were similar to previously reported pharmacokinetic parameters for both mice and rats.\textsuperscript{19,20} \textsuperscript{b}p < 0.05.

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Figure 5. Bioluminescent images of mice treated by saline (control), camptothecin salt solution (CPT-Na), and camptothecin nanocrystals (CPT-NCs), respectively, that were measured at the end of the efficacy study (A); and quantification of the total tumor metastasis burden in mice (n = 10; mean ± SD) by the bioluminescent, luciferase activity measured in p/s·cm$^{-2}$·sr (B).
Tumor tissues treated with CPT nanocrystals induced the most apoptotic cells, while only a few scattered TUNEL-positive cells were visible from the treatment by the CPT salt solution. The control group, receiving saline, had a negligible effect on apoptosis of tumor cells. Apparently, the nanocrystals effected the most drastic antitumor activity against the tumor cells in the animals. One likely reason is much larger accumulation by the nanocrystals in the lung than the drug solution, although this has not yet been confirmed. In our previous study, the AUC\textsubscript{0–24} of drug in lung of CPT nanocrystals was 7.5 times higher than that of the salt solution in a similar murine tumor model.\textsuperscript{12} Moreover, because of the poor solubility of the drug, the accumulated nanocrystals in the lung likely released free drug molecules in a slow and sustained fashion, significantly increasing the drug exposure to cancer cells. We were not able to detect the expected dissolution of the drug in the plasma, but this may be explained by the low concentration achieved being below the LOQ for our assay. Also, it is also possible that the mice achieve difference in pharmacokinetics compared to rats, which were used to determine our pharmacokinetic parameters. It is possible that the nanocrystals distributed and accumulate quite differently in the different animals, but previous results clearly indicate that the pharmacokinetics of camptothecin solutions are similar in rats and mice.\textsuperscript{19,20}

![Figure 6. Representative confocal images tumor samples in nude mice treated with 0.9% saline (A1–3), camptothecin salts solution (B1–3), or camptothecin nanocrystals (C1–3). Apoptotic cells were labeled with TMR red (red; panel 2 and 3) and nuclei of all cells (live and dead) were stained with Hoechst 33258 (blue, panel 1 and 3). The panel 3 (A3, B3, and C3) is the merged image of panels 1 and 2.](image)

What is equally important for the treatment efficacy by the nanocrystals perhaps comes from the chemical stability that is achieved in the solid-state form. Most of drug molecules in the salt solution already converted to the inactive carboxylate form at the physiological pH.\textsuperscript{22} On the other hand, drug molecules inside nanocrystals remain intact until they are released into a liquid milieu.

A survival study was further conducted to evaluate the treatment efficacy against lung metastasis by the CPT nanocrystals. Mice that developed metastases in the lung were treated by saline, CPT salt solution, and CPT nanocrystals, respectively, with a single injection on days 0, 3, and 6 (Figure 7A). The average survival periods of the animals were 18.4, 21.9, and 30.8 days for the groups treated by saline, CPT salt solution, and CPT nanocrystals, respectively. The nanocrystal treatment significantly increased the life span of the mice when compared with the solution (\(p < 0.05\)) and saline (\(p < 0.005\)). The solution formulation prolonged the survival of the animals slightly, but not significantly, compared with the control group (\(p > 0.1\)). In addition, the body weights of the animals were monitored during the survival study (Figure 7B). The mice suffered slight weight loss during the treatment by the nanocrystals, but their weights recovered after day 9. The mice in the control and salt solution groups grew slowly after a similar refractory period. There was no significant difference in body weight among the treatment groups, suggesting that the nanocrystals were well-tolerated by the animals.

In summary, camptothecin nanocrystals were developed by solution crystallization and tested in vivo for treating lung metastasis. The pharmacokinetic metrics indicated a prolonged circulation by the nanocrystals in blood and significantly improved treatment efficacy as compared with a salt solution formulation. The animals that received the nanocrystal treatment endured an extended life span. It is believed that the nanocrystals accumulated in the lung, possibly due to the needle-shaped morphology and the particle size ranging from 200 to 700 nm and thereby presented a great exposure of free drug molecules to cancer cells over the experimental duration as the nanocrystals slowly dissolved. While the size and shape factors have been shown for lung targeting by drug delivery systems,\textsuperscript{23,24} further...
studies are warranted for understanding the lung accumulation mechanism of drug nanocrystals. More importantly, the nanocrystals protected the active form of the drug from chemical degradation in solution not only enhancing the anticancer efficacy but also minimizing systemic toxicities. The salt solution, conversely, relied on the conversion from the delivered inactive form. For treating lung metastases, CPT nanocrystals may be viable option, deserving further optimization and investigation.

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**Notes**
The authors declare no competing financial interest.

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Figure 7. Kaplan−Meier survival curve of mice bearing MDA-MB-231/Luc tumors after treated with saline (control), drug salt solution (CPT-Na), and nanocrystals (CPT-NCs), respectively. The results were significant ($p < 0.05$ for CPT-NCs vs CPT-Na and $p < 0.005$ CPT-NCs vs control) (A). The body weight change of the groups during the first 18 days of treatment, in which the injections are marked by red arrows (B).